CHEMOTHERAPEUTIC POTENTIAL OF CURCUMIN IN ORAL CANCER AND ITS POTENTIATION OF CISPLATIN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE M.S. BRANCH 1 (GENERAL SURGERY) EXAMINATION OF THE TAMIL NADU DR.M.G.R. MEDICAL UNIVERSITY, CHENNAI, TO BE HELD IN APRIL 2013.

CERTIFICATE

This is to certify that the dissertation entitled **"Curcumin potentiates the chemotherapeutic efficacy of Cisplatin in 7,12 dimethylbenzanthracene induced oral cancer in Syrian golden hamsters"** is a bonafide work by Dr.Gayatri Balachandran, submitted in partial fulfilment of the requirements for the M.S. General Surgery (Branch I) examination of the Tamil Nadu Dr.M.G.R. Medical University, Chennai, to be held in April 2013.

Dr.John.C.Muthusami	Dr.Pranay Gaikwad	Dr.Benjamin Perakath
Professor (on leave),	Professor and Acting Head,	Head of Department,
Surgery I,	Surgery I,	Dept of Surgery,
CMC, Vellore.	CMC, Vellore.	CMC,Vellore.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my guides Dr.John Muthusami and Dr.Pranay Gaikwad for having faith in the concept of this thesis and for their unrelenting support throughout the process of its completion.

I must thank all the staff and attenders of the Animal House in Welcome laboratory, CMC Vellore, especially Mr.Antony and Dr.Sumathi.

I take this opportunity to thank the Department of Surgery, CMC Vellore, for the opportunity to complete this dissertation, and for all the encouragement I have received for the same.

On a personal note I must thank my friends who were there for moral and physical support, right from the time of drafting the proposal to finally writing the dissertation. It was a mammoth effort, which I could not have pulled off without you.

To my family, in Trivandrum and Bangalore, a big thank you, words just cannot suffice.

Thanks, last but not least, to God, for taking me through till the end.

CONTENTS

INTRODUCTION	**2**
AIMS AND OBJECTIVES	**5**
BACKGROUND AND LITERATURE REVIEW	**7**
MATERIALS AND METHODS	**40**
RESULTS	**48**
DISCUSSION	**61**
LIMITATIONS	**72**
CONCLUSION	**74**
BIBLIOGRAPHY	**76**
ANNEXURES	**80**

INTRODUCTION

TITLE OF THE ABSTRACT : Curcumin exerts a chemotherapeutic effect and potentiates that of Cispatin in 7,12 dimethylbenzanthracene induced cancer in hamster buccal pouch.

DEPARTMENT : Surgery Unit I, Department of General Surgery, CMC Vellore.

NAME OF THE CANDIDATE: Dr. Gayatri Balachandran

DEGREE AND SUBJECT : MS, General Surgery

NAME OF THE GUIDE :Dr. Pranay Gaikwad and Dr.John C Muthusami (on leave)

TITLE :

Curcumin exerts a chemotherapeutic effect and potentiates that of Cispatin in 7,12 dimethylbenzanthracene induced cancer in hamster buccal pouch.

OBJECTIVES:

The objective of this study was to induce oral squamous cell carcinoma in Syrian golden hamsters with 7, 12 dimethylbenzanthracene and to measure the reduction in tumour area and gross tumour volume and proliferation of the tumour following oral administration of Curcumin, and measure its potentiating action on that of parenterally administered Cisplatin.

METHODS:

A comparative animal study was designed using Syrian golden hamsters. There were four study arms with 6 animals each, adding upto 24 animals. 7, 12 dimethylbenzanthracene was employed as a topical carcinogen, applied to the buccal pouch of all 24 hamsters thrice a week for 12 weeks. Following tumour induction, the subjects were divided into four groups as follows:

A – Control group
B – Curcumin alone group
C – Cisplatin alone group
D – Curcumin along with Cisplatin group

Curcumin was administered enterally at a dosage of 80mg/kg/day for six weeks, while Cisplatin was delivered by intraperitoneal injection once a week for four

weeks, each dose amounting to 40 mg/m^2 (body surface area). Following this, the animals were sacrificed and the buccal pouch submitted for microscopic analysis.
The outcomes studied were absolute and percentage reduction in tumour area (from pre- to post-intervention), tumour volume post-intervention and proliferative index of the tumour based on mib-1 staining on histology.

RESULTS:

There was 100% tumour incidence in the control arm. The maximum reduction in area of tumour was in Group D (77.22%), as compared to 47.82% in Group C and 16.08% in Group B. The interaction was, thus, synergistic. This difference was statistically significant (p value <<<). The lowest tumour volume and lowest proliferative index on histology was also observed in Group D, proving that maximum tumour inhibition was achieved with combination therapy as opposed to either agent alone.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is among the top three cancers in India, with a prevalence of more than 30 %.(1) The most consistently identified risk factor is the abuse of tobacco, both in its smoked and smokeless forms. The magnitude of this cancer is on the rise, and its importance as a leading cause of cancer-related death has expanded its scope to a global dimension. In India, oral cancers form the preponderant cancer among this group of HNSCC.(2)

The increasing burden of disease demands prompt diagnosis and early initiation of treatment, which is at the same time of standard quality as well as accessible and available to the alarming number in need of it. The standard management protocol includes radical surgery along with high dose radiochemotherapy. The morbidity associated with each of these modalities has been well-documented.

The problem that is faced in expediting treatment is in fact delayed diagnosis. The disease is most commonly an affliction of the lower socioeconomic strata of society, and that too predominantly of the rural population in whom tobacco

practices run high. Presentation to a health centre is often delayed, hence disease is usually advanced at presentation. Later the stage of diagnosis, lower are the expected treatment outcomes and greater are the costs expected to be incurred for more radical multidisciplinary treatment.(3)

With the new understanding of nutri-therapeutics and application of traditional medicine to the treatment of cancer, efforts are underway to arrive at a new approach. It is with this regard that we have proceeded to investigate the role of turmeric, a time-tested herbal remedy with mysterious therapeutic properties, in the carcinogenesis and tumour regression of this particular malignancy.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

AIM

To explore the chemotherapeutic potential of Curcumin (the active principle in Turmeric) in oral cancer models in hamsters.

OBJECTIVE

To induce oral squamous cell carcinoma in Syrian golden hamsters with 7,12 dimethylbenz(a)anthracene and to measure the reduction in tumour area and gross tumour volume, and to analyse histology and proliferation following oral administration of Curcumin, and measure its potentiating action on that of parenterally administered Cisplatin.

BACKGROUND AND LITERATURE REVIEW

BACKGROUND AND LITERATURE REVIEW

ORAL CANCER

Oral cancer is defined as per the International Classification of Diseases scoring system, wherein the anatomical boundaries of the oral cavity are described.(4) Briefly put, the cancers arising from the lip(ICD 140), mouth and tongue(ICD 141,143 – 145) fall in this category. The histology of interest is of course, squamous cell carcinoma, which forms over 80% of the malignant neoplasms of the orofacial region, if facial skin is excluded.(5)

EPIDEMIOLOGY

On a world wide scale, oral cancer is the tenth highest occurring cancer among men and fourteenth among women.(6) The age-adjusted rates of HNSCC are highest in the wine drinking districts of Southern France, followed by Bhutan, Bangladesh, India, Brazil and among African Americans in the USA. Among women, the age-adjusted rates are highest in the world in Papua New Guinea

and parts of South-Central Asia. (6) In India, the oral cavity is the predominant site, whereas in France it is the hypopharynx. (6) This difference in sub-site predilection is probably due to variations in exposure to risk factors among the population.(6)

Epidemiological studies suggest an increasing trend of incidence of oral carcinoma in India. Data was extracted from the Globacon project 2008, and crude projections for oral cavity cancer from 2008 to 2030 show an alarming increase in incidence of the disease, in both sexes.(6) The estimated oral-cancer related death toll in 2020 in both sexes is almost 70,000.

AETIOLOGY

More than 75 % of oral cancers are attributable to modifiable risk factors. (7) The most prominent among these is tobacco use, both in smoked and smokeless forms. Others include excessive alcohol consumption, poor oral hygiene, chronic irritation and inflammation induced by ill-fitting dentures or sharp teeth

and by chronic infection with Human Papilloma Virus (HPV). Traditionally, conditions like syphilitic glossitis and excessive consumption of spicy food are also thought to be risk factors. There are also reports of increasing incidence of oral squamous cell carcinoma in chronic immunodeficiency states like post-organ transplantation.(8)

Tobacco:

Tobacco, whether smoked, chewed or snorted as snuff, has been categorically proven to induce carcinogenesis, both as an initiator and as a promoter of tumorigenesis. (8)

Alcohol:

Numerous case-control studies have shown significant association between alcohol and risk of oral cancer. (8) The use of alcohol has shown synergistic action with the abuse of tobacco. The exact mechanism of action is still unclear but numerous hypotheses have been put forward. Alcohol is thought to act as a

solvent for carcinogens like those in tobacco. The cytochrome-inducing ability of ethanol may accelerate metabolism of carcinogens in the liver and facilitate their action.(8)

HPV:

Chronic infection with HPV, es[ecially strain 16, is an independent risk factor for oral cancer, specifically oropharyngeal and tonsillar malignancy. The importance of this viral aetiology lies in the fact that in a number of recent epidemiological studies, HPV infection has emerged as a leading risk factor among young adults afflicted with oral cancer, especially among the non-smoking population. (7)

MANAGEMENT

The main public health challenges involved in confronting this disease include lack of reliable screening systems, risk taking behaviour especially among the lower socioeconomic groups resulting in increased exposure to known risk

factors and delayed diagnosis, lack of education and awareness among the at-risk population, and prohibitive costs of treatment.

As per the National Comprehensive Cancer Network guidelines(9), the treatment algorithm for early oral cancers is single modality, either definitive resection or radical radiotherapy. In locally advanced cancers, multidisciplinary treatment is the mandate, requiring a combination of surgery, radiotherapy and in the presence of high risk factors, chemotherapy. In squamous cell cancers of the head and neck, the foremost chemotherapeutic agent, especially for locally advanced or unresectable tumours, remains Cisplatin, alone or in combination with infusional 5-Flurouracil or Paclitaxel.(10)

CISPLATIN

Cisplatin is a platinum based alkylating agent, the first medicine developed in

that drug class. Other drugs in this class include carboplatin and oxaliplatin. Cisplatin is called the “penicillin of cancer” because it is used so widely and it was the first major chemotherapy drug to be developed. Despite the advent of targeted therapies to individual cancers, this old drug still retains its relevance in treatment protocols for a variety of cancers. In the case of HNSCC, the appropriate regimen, whether induction or concurrent chemotherapy, still remains a controversy, but studies in both directions have demonstrated benefit in advanced malignancy, in the form of decreased incidence of distant metastasis and recurrence(10), and definite survival benefit has been demonstrated in a few papers.

The problem with the drug is the side-effect profile which can limit its use. These include:

- *Nephrotoxicity* : The nephrotoxicity of platinum-class drugs is a dose-limiting side effect and seems to be related to reactive oxygen species.(11)
- *Neurotoxicity*

- *Nausea and vomiting*: This is induced by both a central action on the chemotrigger zone as well as a direct action on the intestinal mucosa.
- *Ototoxicity* : This is a permanent effect related to its ability to bind to melanin in the stria vascularis of the inner ear or the generation of reactive oxygen species. It manifests as tinnitus and high frequency hearing loss.(11)
- *Dyselectrolytemia:* Cisplatin can cause hypomagnesaemia, hypokalaemia and hypocalcaemia.(10)
- *Myelotoxicity*
- *Haemolytic anaemia*: It is suggested that an antibody reacting with a cisplatin-red-cell membrane is responsible for haemolysis.

While modern medicine has yet to provide a completely suitable safer alternative for this drug in oral cavity cancers, where can we turn to for one? Back to the "roots" of Pharmacotherapy perhaps.

NUTRICEUTICALS

"Let food be thy medicine and medicine be thy food."

Hippocrates.

Hippocrates said it right when he made this famous statement centuries ago. Little did he know the implications of his words. The application of food-derived substances to the treatment of diseases and optimisation of lifestyle is a vast avenue for study, and one with tremendous potential for research and development. From chronic degenerative diseases to erstwhile incurable malignancies, nutri-therapeutics, as it is called, could hold the key to the next step in management.

Tumourigenesis is a multi-step pathway that involves transformation of a normal cell into an autonomous dysregulated proliferative cell with metastatic potential.(12) Although the mysterious details of this process at a molecular level still form the subject of research today, what we do know is that every step of the process involve alteration of cell signalling pathways. Cancer cells have

possess certain abilities that normal cells do not: resistance to growth inhibition, independent proliferation and limitless replication, immortalization, stimulation of angiogenesis and ability to infiltrate and metastasize.(12) Each of these abilities is controlled by specific genes and signalling pathways involving a number of transduction molecules , thus offering multiple potential targets for therapy. Most of modern day anti-neoplastic agents offer single-target action and are therefore ineffective in single-handedly preventing or treating cancer. Therefore the current philosophy of chemotherapy is to combine several monotargeted drugs or provide drugs that target multiple molecules.

Many plant-derived dietary agents, some used for centuries for their medicinal properties, have multi-targeting properties. This has fuelled the development of a new stream of drug development, called Nutraceuticals.(13) They offer the advantage of being less expensive, with fewer side effects and easier accessibility.(13)

A nutraceutical, coined by Stephen DeFelice in 1989(14), is any substance considered to be food or part of a food that provides medical and health benefits. The study of these therapeutic agents holds promise for a whole new avenue for cancer prevention therapy. Among the broad variety of nutraceuticals that have been investigated, we have focussed on one close to home: curcumin, the active principle of turmeric.

TURMERIC : INDIA'S SOLID GOLD (15)

Turmeric (Curcuma longa Linn.; syn.: Curcuma domestica, Curcuma aromatica) is a perennial rhizome from the Zingiberaceae family that is widely cultivated in the tropical regions of Asia, most extensively in India, and Latin America. Synonyms for this herb include Indian saffron, turmeric root, and yellow root. The applicable part of turmeric is the root, which is rich in potassium and iron.

Figure 1: Turmeric, a perennial rhizome. (From www.turmeric-curcumin.com)

Turmeric has been exploited as a medicinal herb for centuries, forming a part of the armamentarium of Ayurvedic medicine. It is an essential spice, forming a component of Indian cuisine and has been used for its preservative properties. Turmeric contains numerous phytochemicals, including curcumin. It is now recognised that it is curcumin that lends the yellow colour to the spice and is responsible for its therapeutic properties.(16)

Curcumin was first isolated in 1815 and its chemical structure was identified to be diferuloylmethane (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) in 1920. It exists as three analogues: curcumin (curcumin I),

demethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III) of which curcumin is thought to be the most biologically active.(17)

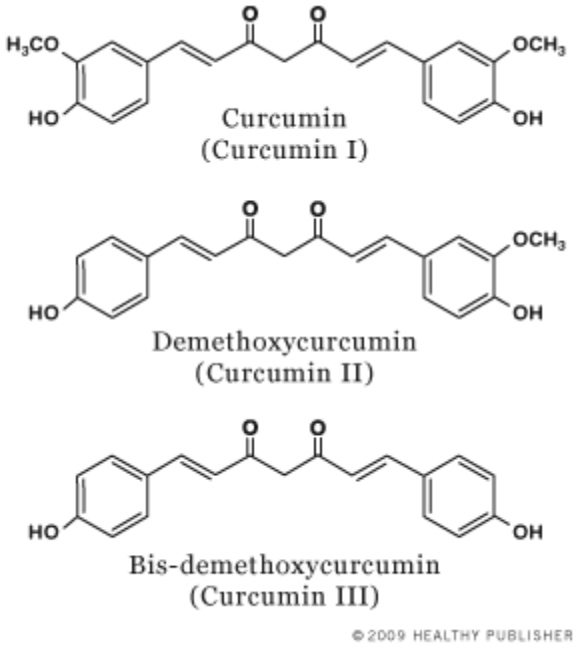


Figure 2: Curcumin exists in three forms, of which Curcumin I is the most abundant.(From www.turmeric-curcumin.org)

The pharmacokinetics of curcumin are yet to be fully characterised. What is known is that curcumin is poorly absorbed orally due to its poor solubility, and its high rate of metabolism in the liver and rapid excretion in bile.(18) During absorption, it is changed into products which are more polar and accumulate on the serosal aspect of the intestine. It is metabolised by glucuronidation to form

curcumin glucoronide and curcumin sulphate, and bioreduction to form tetrahydrocurcumin, hexahydrocurcumin and hexahydrocurcuminol.(19) Metabolites like Tetrahydrocurcumin(THC) also possess anti-inflammatory properties but are less potent that the progenitor.(19) It has been proven scientifically that this biotransformation takes place in the intestines as well as in the liver.(19) This avid intestinal metabolism may also contribute to its poor systemic bioavailability when administered enterally.

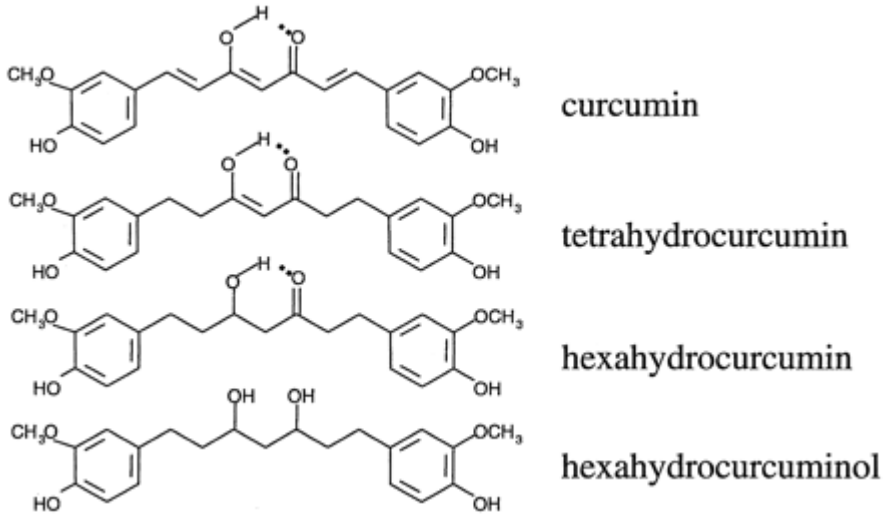


Figure 3: Curcumin and its products of bioreduction(19)

When administered orally, upto 75% can be excreted in faeces, whereas the rest appears in traces in the urine.(18)

The use of turmeric in the treatment of maladies is one known for generations, described in the folklore of Indian and Chinese schools of medicine. The list of uses include anti-inflammatory, antiseptic, analgesic, anti-malarial and for promotion of wound healing.

The basic principle behind its multifaceted utility is hypothesized to be its anti-inflammatory property. Dysregulated inflammation is now thought to be the key step in many a chronic illness and in carcinogenesis itself(20). Studies have shown that curcumin targets many inflammatory mediators, critically Tumour Necrosis Factor α (TNFα) (19) and Nuclear factor κβ (NFκβ).(21) The suppression of activation of NFκβ has in particular been linked to most of its anti-inflammatory and in turn anti-neoplastic effects. (Fig4.)

There is a large body of work reflecting the application of curcumin in chronic neurodegenerative, cardiovascular and rheumatic disorders, the details of which are beyond the scope of this present discussion. The diagram below summarises

the various therapeutic applications of Curcumin (Fig 5). The picture is

borrowed from Aggarwal, et al.(20)

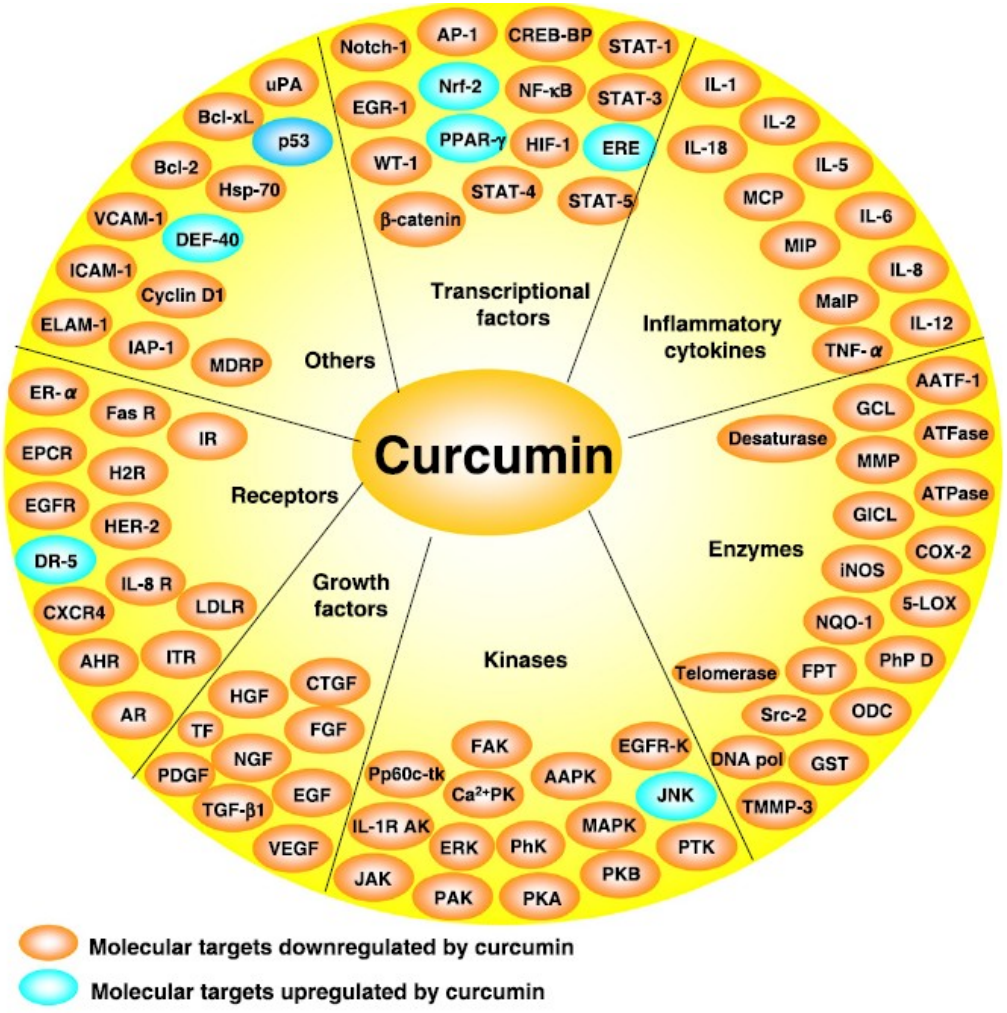


Figure 4: Molecular targets of curcumin : NF-kB, nuclear factor-kappa B; AP-1, activating protein1; STAT, signal transducers and activators of transcription; Nrf-2, nuclear factor 2-related factor; Egr-1, early growth response gene-1; PPAR-c,peroxisome proliferator-activated receptor-gamma; CBP, CREB-binding protein; EpRE; CTGF, connective tissue growth factor; EGF, epidermal growth factor; EGFRK, epidermal growth factor receptor-kinase; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; TGF-b1, transforming growth factor-b1; VEGF, vascular endothelial growth factor; AR, androgen receptor; Arh-R, aryl hydrocarbon receptor; DR-5, death receptor-5; EGF-R, epidermal growth factor-receptor; EPC-R, endothelial protein C-receptor; ER-a, estrogen receptor-alpha; Fas-R, Fas receptor; H2-R, histamine (2)-receptor; InsP3-R, inositol 1,4,5-triphosphate receptor; IR, integrin receptor; IL-8-R, interleukin 8-receptor; LDL-R, low density lipoprotein–receptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase-3; iNOS, inducible nitric oxide oxidase; COX-2, cyclooxygenase-2; LOX, lipoxygenase; Gcl, glutamate-cysteine ligase; NAT, arylamine N-acetyltransferases; IAP, inhibitory apoptosis protein; HSP-70, heat-shock protein 70; TNF-a, tumor necrosis factor alpha; IL, interleukin; MCP,

monocyte chemoattractant protein; MIF, migration inhibition protein; MIP, macrophage inflammatory protein; ERK, extracellular receptor kinase; IARK, IL-1 receptor-associated kinase; cAK, autophosphorylation-activated protein kinase; CDPK, Ca2+-dependent protein kinase; cPK, protamine kinase; JAK, janus kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TK, protein tyrosine kinase; FAK, focal adhesion kinase; PhK, phosphorylase kinase; pp60c-src, pp60c-src tyrosine kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; FPTase, farnesyl protein transferase; GST, glutathione S-transferase; HO, hemeoxygenase; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ELAM-1, endothelial leukocyte adhesion molecule-1; SHP-2, Src homology 2 domain-containing tyrosine phosphatase 2, uPA, urokinase-type plasminogen activator.(20)

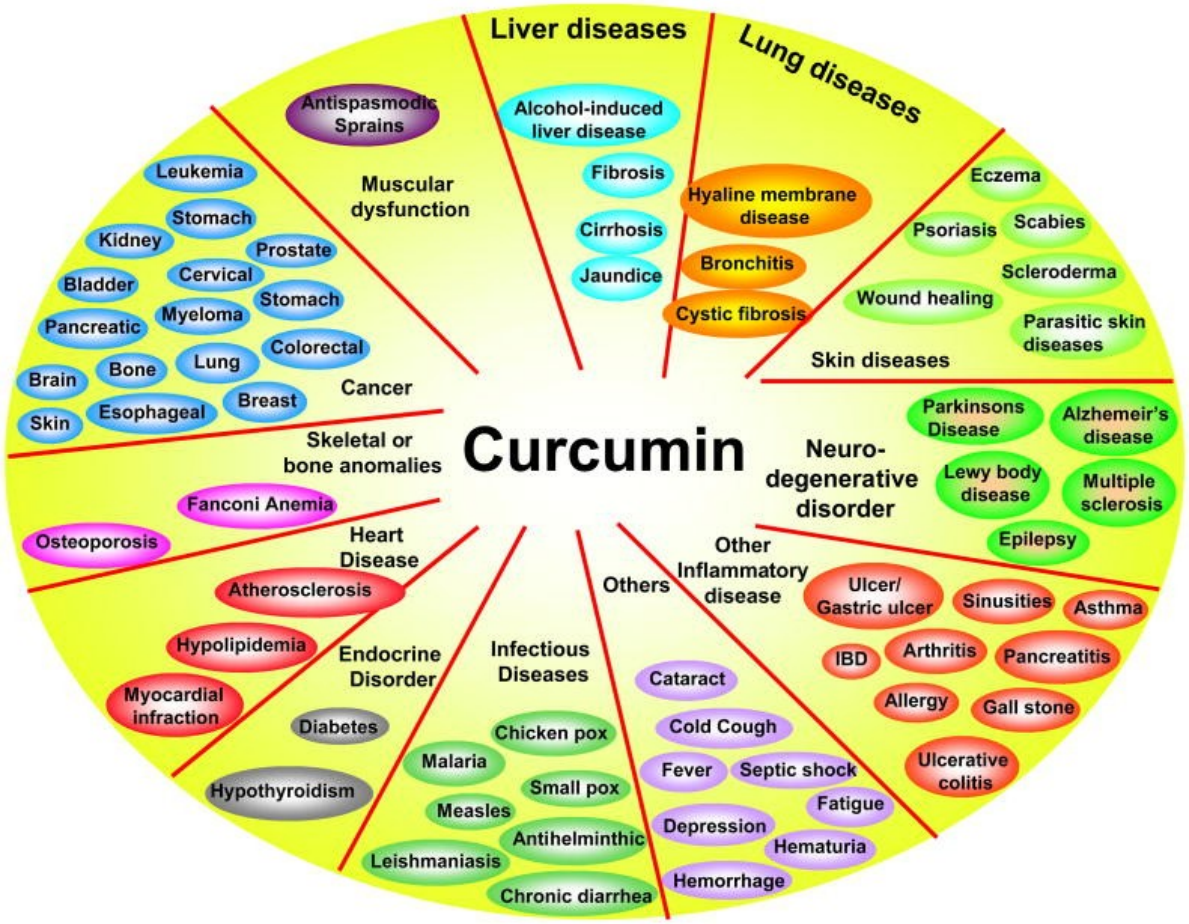


Figure 5 Curcumin and its application in various pro--inflammatory conditions(20)

CURCUMIN AND CANCER

There have been more than 800 *in vitro* and *in vivo* studies demonstrating the anti-cancer potential of curcumin(20,22). Every step of the tumourigenesis pathway, i.e. tumour initiation, progression, angiogenesis and evasion of cell death have been shown to be targeted and downregulated by curcumin through various molecular targets including transcription factors, growth factors, receptors thereof, cytokines, enzymes and genes regulating cell proliferation and apoptosis. The pro-apoptotic property of curcumin is both a direct action as well as through signalling pathways involving the above mentioned molecule. The specific molecular targets involved are outlined below in Fig 6.(22) Because of numerous mechanisms used by curcumin, it is possible that cells may not develop resistance to curcumin-induced cell death.

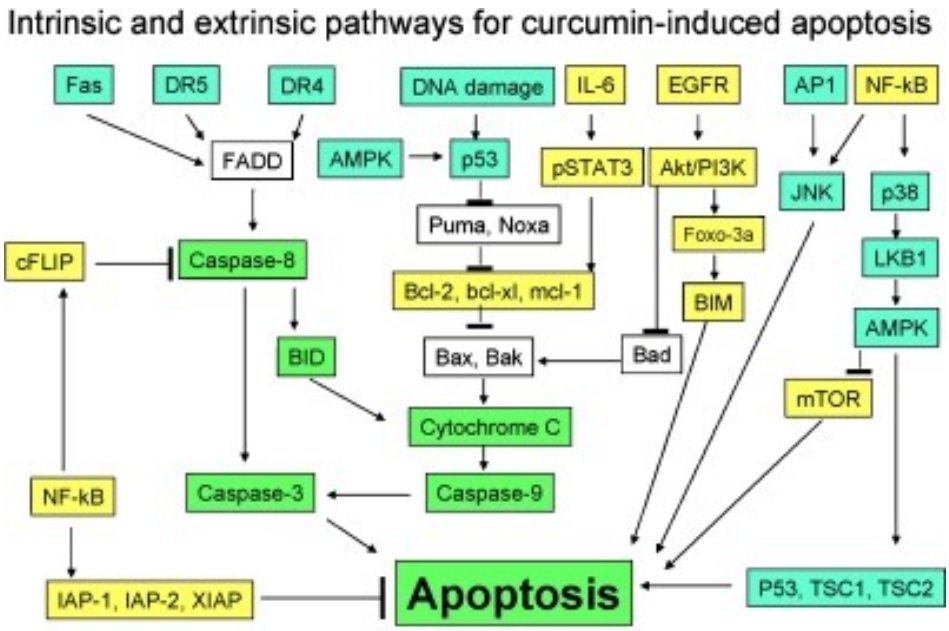


Figure 6: Modulation of various cell death pathways by curcumin. Targets up-regulated by curcumin are in a blue box, those down-regulated are in a yellow box, and those unaffected are in a white box. AP-1 activator protein-1, AMPK 5' adenosine monophosphate-activated protein kinase, *BID* BH3 interacting domain death agonist, *BIM* BCL2-like 11 (apoptosis facilitator), *cFLIP* cellular FLICE-like inhibitory protein, *FADD* Fas-associated protein with Death Domain, *DR4* death receptor 4, *DR5* death receptor 5, *EGFR* epithelial growth factor receptor, *IAP* inhibitor of apoptosis protein, *IL-6* interleukin-6, *JNK* c-Jun N-terminal kinase, *mTOR* mammalian target of rapamycin, *NF-kB* nuclear factor-kB, *PI3K*phosphoinositide 3-kinase, *STAT3* signal transducer and activator of transcription 3, *XIAP* X-linked IAP (22)

On normal cells, curcumin has found to have minimal if no effect.(22) It is thought that normal cells have less absorptive capacity to curcumin as compared to tumour cells. Glutathione levels in cancer cells are lower than those in normal cells and this has found to increase their susceptibility to curcumin. As alluded to earlier, NF-κβ, a main molecular target of curcumin is expressed constitutively in cancer cells, but not in normal cells. By suppressing the NF-κβ-

regulated products, curcumin can modulate tumour cell proliferation and survival while not affecting normal cells devoid of this molecule. Some studies have demonstrated isolated actions of curcumin on normal cell lines. Human epidermal keratinocytes treated with curcumin have demonstrated apoptosis, through the inhibition of Activator Protein-1. (23) Curcumin in low concentrations decreases the rate of lipid peroxidation and cytochrome c release in human hepatocytes and is therefore cytoprotective. However in higher concentrations, studies have reported glutathione depletion, activation of caspases and direct hepatotoxicity. Increased apoptotic rates have been shown in fibroblasts, endothelial cells, human retinal endothelial cells and lymphocytes. While all these have been demonstrated in laboratory settings, Phase I and II human studies have demonstrated good tolerability with minimal side effects(24). This ability to kill cancer cells preferentially to normal cells makes curcumin an attractive candidate for anti-cancer drug development.

A detailed review of the application of curcumin to human cancers is perhaps beyond the scope of our present discussion. It has been studied in the

management of numerous pathologies, as diagrammatically represented in Fig7(20). Curcumin has been shown to exhibit chemopreventive and chemotherapeutic potential against variety of different cancers including gastrointestinal cancers, genitourinary cancers, melanoma, neurological cancers, leukemia and lymphoma; breast cancer, ovarian cancer, head and neck squamous cell carcinoma, lung cancer and sarcoma.

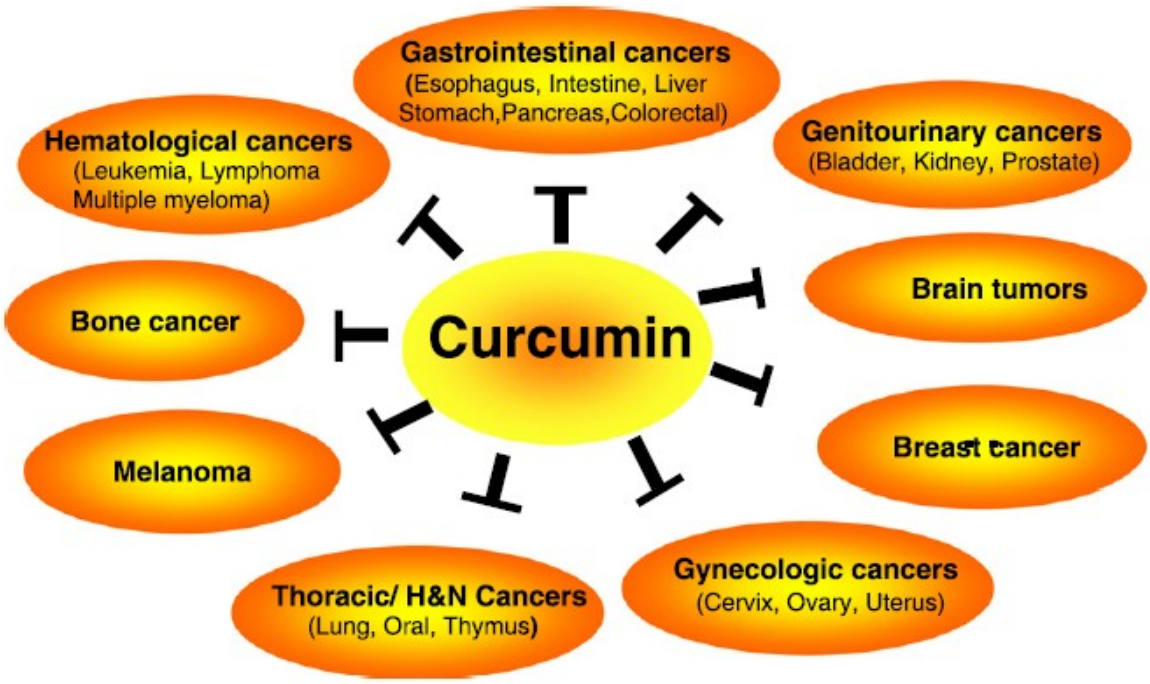


Figure 7: Various cancers against which curcumin has found to have therapeutic potential.(20)

Curcumin has also been exploited in the control of various treatment and disease related symptoms.(20) These include depression, malaise, neuropathic pain, anorexia and insomnia. Most of these symptoms are related to disruption of cytokine homeostasis secondary to chemotherapy. This has been demonstrated in animal experiments where disturbances in levels of pro-inflammatory cytokines like TNFα, IL-1 and IL-6 paralleled changes in "sickness behaviour"(25) like weight loss, loss of appetite and disturbed sleep rhythms. Since curcumin is known to target and down-regulate these inflammatory mediators, it has been tried as an ancilliary treatment to palliate these symptoms.

CURCUMIN IN ORAL CANCER

Curcumin has been shown to inhibit the formation and growth of oral cancer cell lines *in vitro*.(26, 27) Numerous *in vivo* studies using oral cancer models in rats and hamsters have also revealed the efficacy of curcumin, mainly as a chemopreventive agent. In one particular study by Manorahan et al(28), the

chemopreventive effect of curcumin was studied in induced squamous cell cancers of the buccal pouch in hamsters, along with that of piperine. Oral squamous cell carcinoma was induced in hamsters by painting the cheek with a known carcinogen, 0.5% 7,12 dimethyl benzanthracene three times a week for 14 weeks. The animals had been randomized as to receving no concurrent treatment with curcumin or piperine, or treated alone with each. The total number of tumours was determined macroscopically and the buccal pouch was also assessed microscopically. While they observed 100 % tumour formation in the control arm, the formation of tumour was completely prevented in the groups treated with curcumin, as well as with piperine.

A few studies have also assessed the chemotherapeutic effect on oral cancer, both on cell lines *in vitro* models as well as *in vivo*.(29,30) One such study (31) demonstrated the effect of topical curcumin in oral mucosal tumours induced in hamsters. The number and volume of visible lesions reduced by almost 40% and 60%, respectively. Tumour angiogenesis was also inhibited, along with decrease in proliferative index and dysplasia.

There are few trials that have tried the drug in human beings for oral cancer. In a Phase I clinical trial on high risk premalignant lesions,(29) 7 patients with oral leucoplakia were treated with serially incremented doses of curcumin for 3 months. The maximum tolerable dose was found to be 8g/day. 2 out of the 7 patients showed significant histological improvement.

CURCUMIN : A CHEMO-RADIOTHERAPY SENSITISER

In addition to its evident anticancer activity both in prevention and therapy, curcumin has also been postulated to offer a chemosensitising and radiosensitising role in the treatment of malignancy. In the age of targeted therapies and danger of resistance among cancer cells, curcumin may offer a way out: a mulitargeted therapy with minimum toxicity and with an ability to increase apoptotic rates to standard chemoradiotherapy regimens and thereby reduce generation of resistant cancer cell lines. In a review by Goel and

Aggarwal (32), they analysed both *in vitro* and *in vivo* studies assessing this ability of curcumin in a variety of cancers.

In the realm of oral squamous cell cancer however they have mentioned a study by Khafif et al(33). Squamous carcinoma cell lines were pre-treated with curcumin, and then subjected to doses of radiation. This resulted in significantly decreased cell growth and reduced ability for subsequent tumour colony formation in response to ionizing radiation therapy. In a more recent experiment,(34) the synergistic effect of curcumin and irradiation was demonstrated in a similar *in vitro* setting.

In an experiment by Duarte *et al* (35) the additive effect of curcumin along with Cisplatin was studied using two squamous carcinoma cell lines CAL27 and UM-SCC1. The study encompassed an *in vitro* analysis of suppression of tumour cell proliferation as well as an *in vivo* study using the same cell lines xenografted onto nude mice. Intravenous tail vein injection of liposomal curcumin and intraperitoneal Cisplatin were administered alone or in combination to the mice

growing the clinically evident tumours. They were able to demonstrate a greater tumour suppression with the combination as compared to either agent alone, in both *in vitro* and *in vivo*. But their results were not statistically significant in the xenograft model.

Suh, et al (36) performed an experiment with similar methodology, in which a 30% reduction in tumour size was demonstrated with a combination of cisplatn and curcumin, a value with statistical significance when compared to the control and with each agent alone.

CURCUMIN AS A CHEMO-RADIOPROTECTOR

Inhibition of pro-inflammatory mediators like NFκβ and suppressing the translation of their target genes is the principle mechanism of most chemotherapeutic agents. However there action on normal cells in addition to cancerous ones is the cause for drug-related toxicity.

The main limitation of cisplatin, as mentioned earlier is the dose-dependent nephro-toxicity, among other crippling side-effects. Reports have shown that

cisplatin-induced renal failure may be due to oxidative stress and could represent a pro-inflammatory reaction to the drug.(32) Curcumin, by way of its potent anti-inflammatory action, has been studied as a potential tool to alleviate the renal damage caused by cisplatin.

Kuhad. et al published an animal experiment in which they replicated cisplatin-induced renal tubular injury and studied the effect of curcumin. They noted that not only did curcumin improve subsequent renal function, but also reversed signs of oxidative stress like lipid peroxidation and decreased levels of inflammatory mediators in the renal tissue.(37)

There is also evidence to suggest a similar protective role following radiotherapy. Numerous *in vitro* and *in vivo* studies (37) have demonstrated that curcumin protects from radiation induced DNA damage in human intestinal cells, blood cells as well as reduces the incidence of pulmonary and cardiac toxicity secondary to whole body irradiation. The negative effect of radiation on wound healing has also been tackled with curcumin. Treatment with curcumin prior to irradiation improved rates of wound contraction, hastened the process of

complete wound healing and also reduced the rate of radiation-induced skin damage.

HAMSTER MODEL FOR ORAL CANCER

The most extensively studied *in vivo* model for oral cancer has been with hamsters. Hamsters have been thought to be the ideal oral cancer model because anatomically they possess a large buccal pouch which offers an accessible relatively large surface area for study. Furthermore, oral ancer when induced in the hamser cheek model, the resultant tumour was sufficiently similar, both histologically as well as behaviourally, to human cancers to justify scientific investigation of the same. Therefore the results are often extrapolated relatively accurately to the human scenario.

The most commonly used animals are Syrian Golden Hamsters (*Mesocricetus auratus)*. These animals average a life span of two to three years. Adults are from 13- 18 cm long and are predominantly nocturnal in habit.(38) They, like

most members of the hamster subfamily possess an expandable cheek pouch that extends from the cheek to the shoulder.

CARCINOGENESIS : REPLICATING THE PATHOLOGY

Carcinogenesis or oncogenesis is the process of transformation of a normal cell into a cancer cell. At a cellular level, the process involves a molecular reprogramming, which ultimately results in loss of regulated cell division, uncontrolled proliferation, preferential consumption of resources, transgression of neighbouring cellular structures and ultimately distant spread. The normal process of cell division is tightly regulated be a number of genes, collectively known as tumour suppressor genes, and in order to maintain homeostasis this is finely balanced with the process of cell death or apoptosis, which is in turn regulated by another set of pro- and anti-apoptotic genes. Natural mechanisms are in play to repair any flaws that are bound to occur in such a precariously balanced system. So the process of cancer generation involves accumulation of

mutations at all these various levels i.e tumour suppressor gene inactivation, alteration of apoptotic genes and dysfunction of DNA repair genes.(39) Tumour promoting areas of the genome i.e protooncogenes, which are in a normal sate of dormancy are in turn activated in a domino fashion. The result is the evolution of autonomous rapidly dividing cellular clones, capable of uncontrolled replication and that are virtually immortal. By the theory of natural selection, these cells survive in preference to normal ones, and do so at their expense.

There are many ways to study the process of carcinogenesis, to replicate it for the trial of therapies and to evolve preventive strategies. One method of induction of cancers spontaneously by the use of chemical treatments, genetic alterations or exposure to environmental factors. This offers the advantage of providing a comparable living environment as in humans, so that researchers may better understand the basis of cancer formation, the influence of the native tissue reaction if any, and through the same means highlight any differences between the animal and human phenotype.(40)

The other method is by culturing known cell lineages of different types of cancer in an *in vitro* method and then inoculating the cells onto a host animal, where they may take root and grow, akin to a parasite. The fundamental difference between the two methods is that in the latter cancerous cells are generated a in a controlled environment and are likely to be monoclonal, each identical to the next and all similar points in the pathway of oncogenesis.

For the purpose of scientific enquiry into carcinogenesis and treatment thereof, numerous synthetic carcinogenic agents have been employed. The one such agent that we have utilised for this study is 7,12 dimethylbenzanthracene (7-12 DMBA). Dimethylbenzanthracene is a polycyclic aromatic hydrocarbon and is one of the most ubiquitous toxic environmental carcinogen.(41) It is lipid-soluble and is concentrated in biological membranes. It has been widely used as a laboratory carcinogenic agent for the synthesis of cancers of oral mucosa, mammary tissue, intestinal mucosa and skin to name a few.

7,12 DMBA is oxidised by P450 enzymes and the metabolites thus formed, react with DNA to form covalent adducts and depurinated abasic sites within the DNA molecule. These acts as tumour initators, and instigate the process of tumour formation.(42)

Its use in oral squamous cell cancer has been documented in many studies. Specifically in hamster buccal pouch models, its application in a standardised regimen over 12-14 weeks (thrice-a-week) has documented 100% tumour formation in control arm (28). Histologically the spectrum of tumour formation extends from papillomas, dysplasia, in situ carcinoma and invasive carcinoma, thus replicating the multi-step carcinogenetic process in human squamous cell carcinoma(28). This offers a definite advantage to *in vitro* studies or xenograft studies with cancer cell culture lines in which homogenous carcinoma cells at identical points in the carcinogenetic pathway are analysed.

As a potent carcinogen, its use demands utmost in safety precautions on the part of the investigator. Recommendations call for the use of a laboratory coat, standard nitrile or latex gloves, safety glasses and use of N-100 respirator or a fume-hood.(44)

THE NEXT STEP

At this point, having established to therapeutic and chemosensitizing role of curcumin in oral cancer, we propose to apply this to native oral cancer induced chemically in the buccal pouch of Syrian Golden hamsters.

MATERIALS AND METHODS

MATERIALS AND METHODS

A comparative animal study was designed using Syrian Golden hamsters. In consultation with the Clinical Epidemiology Unit of Christian Medical College, Vellore and numerous veterans of animal study experts, a sample size was arrived at which provided for six animals per study arm.

After drafting the study proposal, it was approved by the Institutional Review Board of Christian Medical College, Vellore. Subsequently, the proposal was put forward to the Institutional Animal Ethics Committee, where it was approved and the use of Syrian Golden hamsters sanctioned.

24 Syrian golden hamsters, between 4-6 weeks of age, were procured from the National Institute of Nutrition, Hyderabad. All animals were chosen as male, with weight ranging from 80-120 g. They were housed in the Animal Lab at the Williams' building of the Christian Medical College Hospital campus. After an acclimatisation period of six weeks the study was commenced. The veterinary

staff was employed for the upkeep and feeding of the animals. They were nourished with standard issue rodent feed and housed in cages of three each.

The carcinogen of choice, 7, 12 DMBA was purchased from Sigma Aldrich India, along with curcumin and protective equipment. The chemical was procured in solution form, mixed in methanol. The solution was stored at room temperature within a secured area of the Animal facility. Cisplatin was procured from the hospital pharmacy.

CANCER INDUCTION PHASE:

100µL 0.5% 7-12 DMBA solution was painted onto the left buccal pouch of the 24 hamsters using a No 1 paintbrush. No sedation was required for this procedure. Feeds and water were withheld for half an hour to prevent washing away of the chemical from the mucosal surface. This was repeated three times a week for a total of 12 weeks. At the end of this period the animals were divided

into four groups as follows:

A – Control group

B – Curcumin alone group

C – Cisplatin alone group

D – Curcumin along with Cisplatin group

The four groups of six animals each were housed in separate cages for identification.

At the end of Induction the tumour number (N0) was counted and gross tumour area (A0) was assessed by multiplying the values of the two longest mutually perpendicular diameters. Diameters were in turn measured with a linen string and a measuring scale as callipers were difficult to negotiate into the rodent oral cavity.

INTERVENTION PHASE:

Group A: Group A animals received no further intervention and served as controls.

Group B: Group B animals received 80 mg/kg of curcumin daily, admixed in 10 mL of drinking water. The formula was administered by a dropper, delivering the appropriate volume of the fluid. This was continued for six weeks.

Group C: Group C animals were administered 40mg/m2 body surface area of commercially available cisplatin, via an intraperitoneal injection. This was administered once a week for four weeks.

Group D: Group D animals received a combination of the above outlined regimens.

END OF STUDY PERIOD:

Allowing for one week after the last intervention in each arm i.e.; week 5 for Group A and C and week 7 for Groups B and D, the hamsters were sacrificed by thiopentone overdose, under the supervision of the Institutional veterinary personnel. The left buccal pouch was excised in toto till the depth of the underlying muscle.

PATHOLOGICAL EXAMINATION

The specimens thus harvested were fixed in 10% formalin and processed and imbedded with paraffin. 2-3 micrometer sections were cut, mounted onto slides and stained with haematoxylin and eosin for pathologic examination. Additional tests performed included staining with mib1 antibody as a marker for proliferation.

The following were the indicators studied as indicators of degree of malignancy:

1. *Gross tumour volume (V):* This was calculated by assessing the longest three mutually perpendicular diameters and applying the formula: V = $\frac{4}{3}\pi\frac{D1}{2}\frac{D2}{2}\frac{D3}{2}$, where D1, D2 and D3 were the three diameters of the tumour.
2. *Gross tumour area (A1):* This was calculated by multiplying the tumour length and breadth.
3. *Histopathology:* The specific type of lesion was identified and reported; whether papilloma, dysplasia, in situ carcinoma or invasive carcinoma.
4. *Proliferation index:* The number of actively proliferating cells (stained by mib-1 antibody) divided by the total number of epithelial cells counted.
5. *Grade:* The grade of tumour was designated as 1, for well differentiated carcinoma, 2 for moderately differentiated carcinoma, and 3 for poorly differentiated carcinoma.

PRIMARY AND SECONDARY OUTCOMES:

Primary outcome assessed was absolute and percentage reduction in tumour area and comparative tumour volumes among the four arms.

Secondary outcomes included degree of suppression of proliferation, assessed by comparing proliferative indexes in the post- intervention arms.

STATISTICAL ANALYSIS:

The absolute reduction in tumour area, percentage reduction in tumour area, comparative tumour volumes between the four groups, along proliferation index among these groups were compared by the Kruskal-Wallis test. Differences with calculated P values <0.05 were regarded as significant.

RESULTS

RESULTS

Twenty-four hamsters were maintained in our animal laboratory. The average weight at the beginning of the study period was 100g. The induction was commenced after a six week acclimatisation period.

One animal died at the beginning of the 9th week of induction. The cause of death could not be ascertained and the veterinarian felt it could be due to improper feeding, perhaps as a result of inadequate acclimatisation. At the end of the induction period, the average weight of the surviving 23 animals was 92g.

The tumour incidence and size was recorded and values are displayed in Table 1. The median tumour area was 315mm^2 and there were 1.99 tumours per animal.

The animals were separated into the four groups and housed in specially marked cages for the same purpose. Intervention proceeded as detailed earlier. Two more animals expired, one during the 13^{th} week and the other during the 14^{th} week, belonging to group B and group D, respectively.

Animal Number *	Diameter1 (mm)	Diameter2 (mm)	Area of tumour (mm2)	Tumour Number
1	15	12	180	1
3	22	24	528	2
4	18	23	414	3
5	12	15	180	1
6	20	21	420	2
7	23	25	575	3
9	11	13	143	1
10	17	19	323	2
11	18	20	360	2
12	15	16	240	2
13	16	17	272	2
14	18	20	360	3
15	21	25	525	4
16	13	11	143	1
17	11	13	143	1
18	12	13	156	1
19	21	15	315	2
20	21	23	483	3
21	21	23	483	3
22	12	13	156	1
23	12	10	120	1
24	14	12	168	1

Table 1: Dimensions and number of tumours induced in the study animals at the end of twelve weeks of induction with 7,12dimethylbenzanthracene.

* Animal number 2 expired prior to the completion of induction, and therefore those values have been excluded from further study.

Group	Animal Number *	Diameter 1 (mm)	Diameter 2 (mm)	Diameter 3 (mm)	Tumour area (mm^2)	Tumour number
A	1	15	12	2	180	1
	3	22	24	3	528	2
	4	18	23	4	41	3
	5	12	15	3	180	1
	6	20	21	4	420	2
B	8	21	25	1	525	3
	9	10	12	2	120	1
	10	15	17	2	255	2
	11	17	18	3	306	2
	12	13	15	3	195	2
C	13	14	13	1	182	2
	14	15	12	2	180	2
	15	18	20	2	360	4
	16	9	8	2	72	1
	17	8	8	1	64	1
	18	8	9	1	72	1
D	19	12	8	1	96	2
	20	11	10	2	110	2
	21	10	9	2	90	2
	22	6	6	1	36	1
	23	5	5	1	25	1

Table 2: Post intervention diameters including depth of tumour(diameter 3) in mm, followed by tumour area(mm^2) and tumour number.

*Animals 2,7 and 24 expired during the study period and hence have been excluded from further analysis.

Group	Animal number	Tumour Volume (mm^3)	Proliferative index (%)	Grade of tumour
A	1	188.40	24.11	2
	3	828.96	24.16	2
	4	866.64	22.18	2
	5	282.60	22.00	2
	6	879.20	26.12	2
B	8	274.75	22.10	2
	9	125.60	23.50	1
	10	266.90	22.00	2
	11	480.42	20.76	2
	12	306.15	24.88	1
C	13	95.25	22.54	2
	14	188.40	21.76	2
	15	376.80	18.18	1
	16	75.36	19.17	2
	17	33.49	19.44	2
	18	37.68	18.29	1
D	19	50.24	18.12	1
	20	115.13	18.55	1
	21	94.20	18.94	2
	22	18.84	18.28	2
	23	13.08	18.90	2

Table 3: Post intervention tumour volume (mm^3), proliferative index and grade of tumour.
(Grade 1: well differentiated, Grade 2: moderately differentiated, Grade 3: poorly differentiated.)

After sacrifice and final histopathological assessment, the results were as follows (Table 2 and 3). There was 100% tumour incidence in group A, the control arm, proving the carcinogenic potential of 7,12, DMBA.

Among the intervention arms, the reduction in tumour area was expressed as absolute decrease in tumour areas i.e. difference between pre-treatment and post-treatment values in mm^2 (Adiff), as well as a percentage of the original area (Per-diff).

The results showed a decrease in tumour area in Group B of 54 mm^2, 87mm^2 in Group C and a decrease of 219mm^2 in Group D. The results showed a 16.08% decrease in tumour area in animals treated with curcumin alone. In case of animals treated with cisplatin alone, this figure was 49.82%, whereas in Group D, the combination of curcumin and cisplatin produced a 77.22% reduction in

tumour area. The difference between the three groups was found to be statistically significant, when analysed with Kruskal Wallis test.

(p value <<<).

Group	Adiff (mm^2)	PerDiff (%)	Median Tumour volume($mm^{3)}$	Median Proliferative Index
A	0	0	828.9600	24.11
B	54.00	16.0839	274.7500	22.10
C	87.00	49.8252	85.3033	19.30
D	219.00	77.2257	50.2400	18.55

Table 4: Among Groups A, B, C and D, the median reduction in tumour area(Adiff), median percentage reduction in tumour area(PerDiff), median tumour volume and Proliferative Index are shown. Interquartile range is not mentioned.

Similar trends were also observed with median tumour volume. The median tumour volume in the control arm was 828.96mm^3, that of Group B was 274.75 mm^3, that in Group C 85.30 mm^3 and in Group C it was 50.24 mm^3. The difference between the groups was again found to be statistically significant (p value 0.005).

	Adiff (mm^2)	PerDiff (%)	Median Tumour volume(mm^3)
Chi-Square	17.695	19.013	12.663
P value for significance	.001	.000	.005

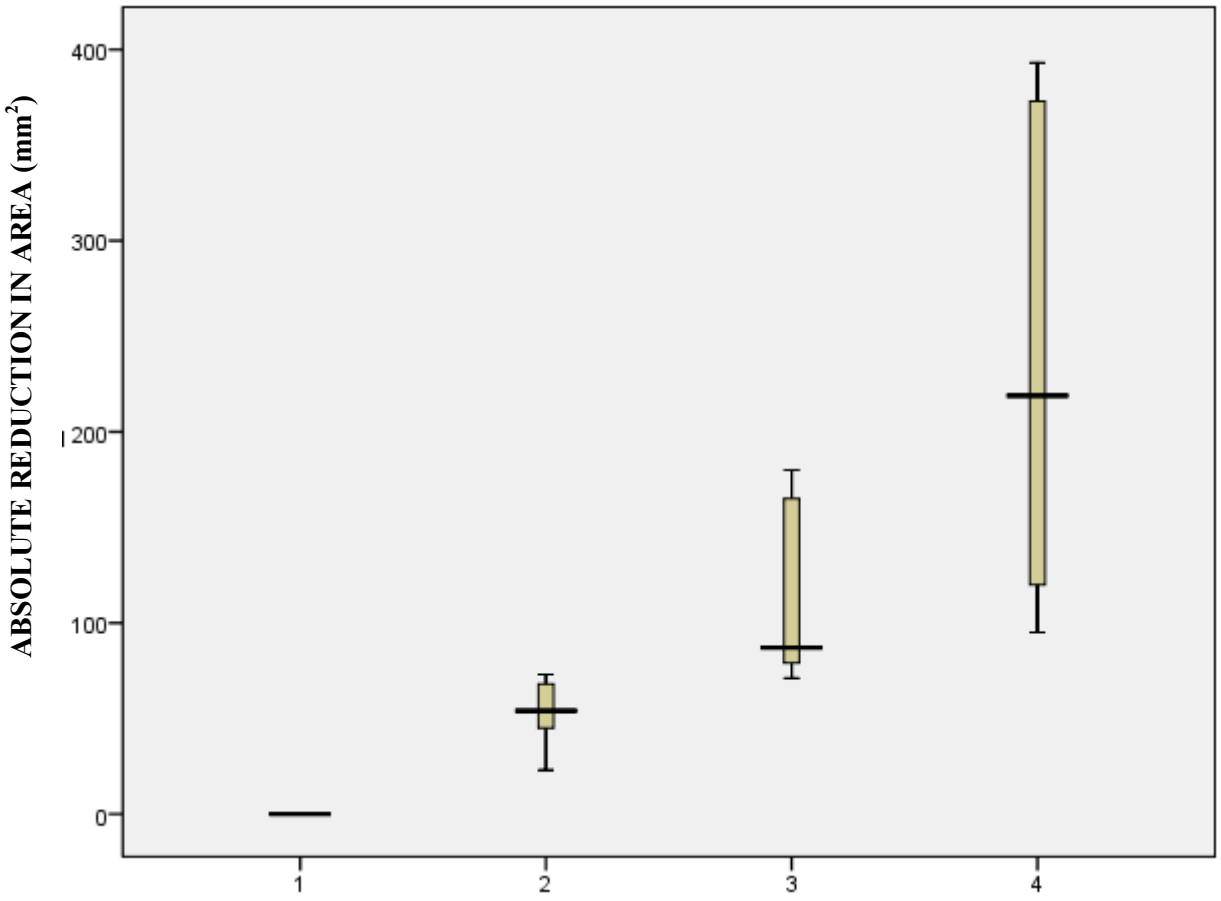
Table 5: Using Kruskal-Wallis test for significance the Chi-square and p values for the three final outcomes are calculated. All values are statistically significant.

The only reduction in gross tumour number was observed in 3 animals, one in Group C and two in Group D. The analysis however was not statistically significant.

Six out of the 21 animals (28.5%) had well differentiated squamous cell carcinoma, and the remaining 71.5% had moderately differentiated malignancy. The median proliferative indices for the four groups are shown in Table 4. On comparing the four group variables using the Kruskal-Wallis test, it was found that the difference between the variables was statisticially significant(Chi square = 13.384 and p value 0.004).

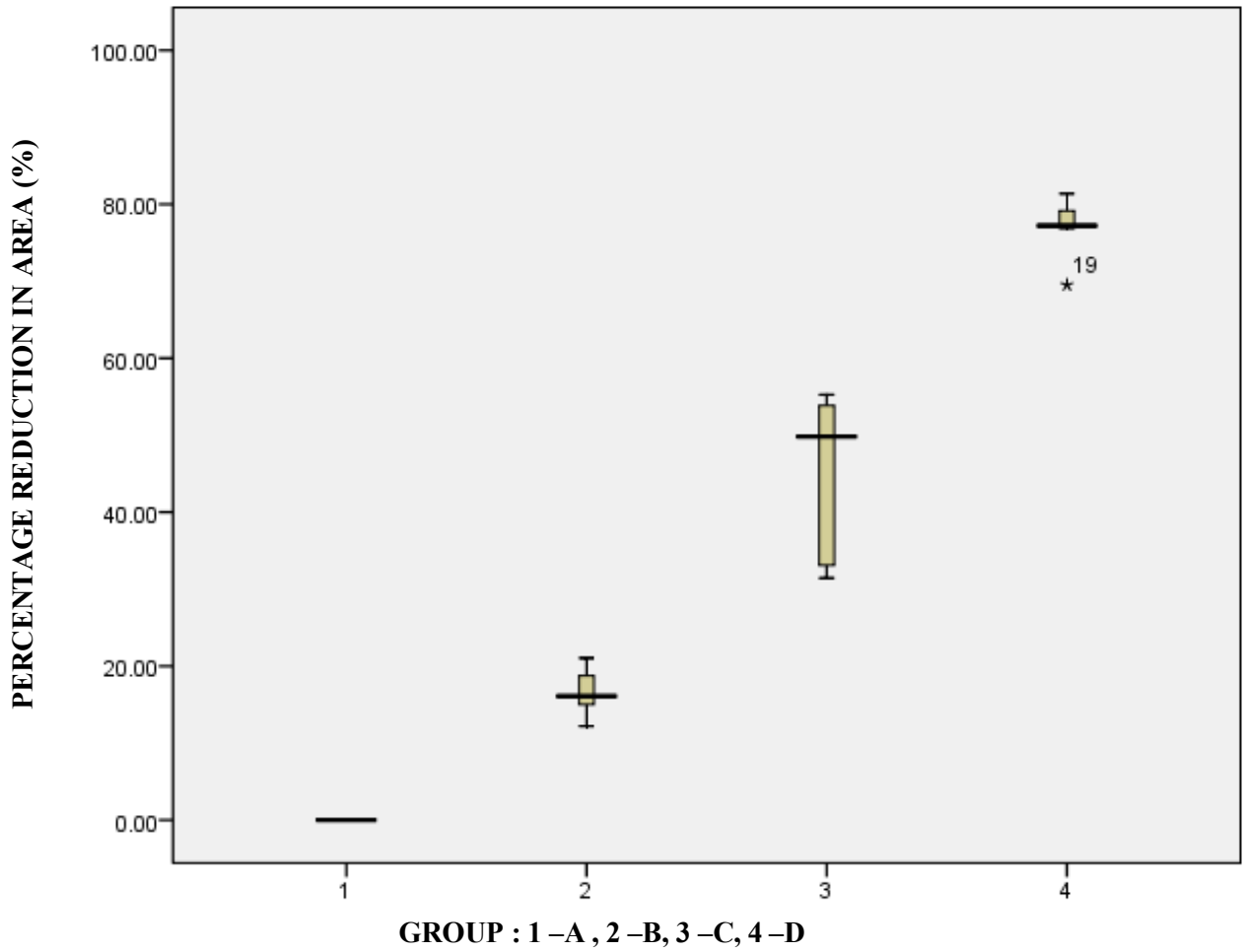
The above results are graphically represented below:.

Graph 1: A diff - Median tumour area reduction from pre-intervenion to post-intervention, in Groups A to D

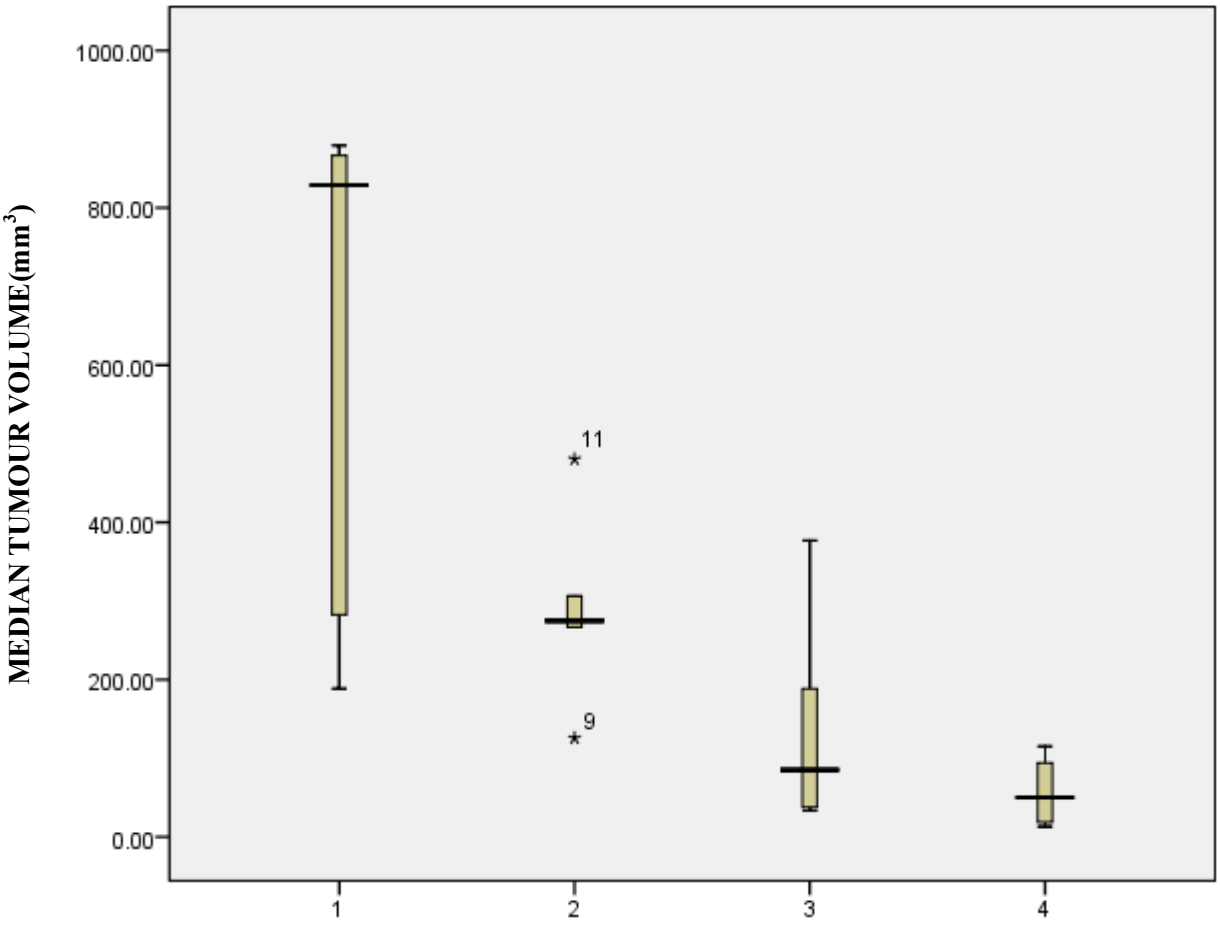


GROUP : 1 –A , 2 –B, 3 –C, 4 –D

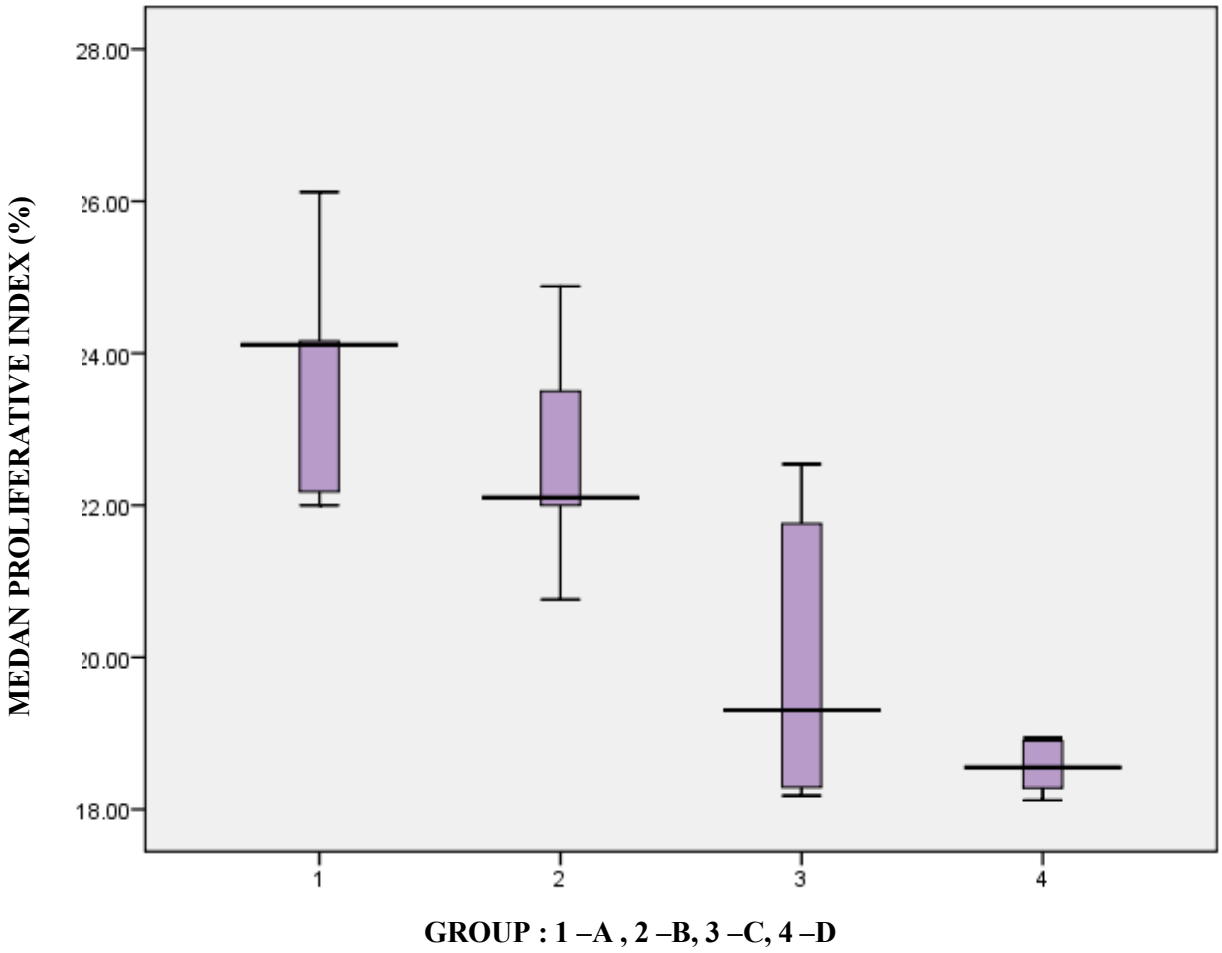
Graph 2: Per-Diff - median percentage reduction in tumour area from pre-intervention to post-intervention in Groups A to D



Graph 3: Median tumour volume post-intervention in Groups A to D



Graph 4: Median Proliferative Index among Groups A to D



DISCUSSION

DISCUSSION

Oral squamous cell carcinoma contributes heavily to the cancer burden in India, and the world. While the mainstay of therapy remains surgery and radiotherapy with chemotherapy for advanced malignancy, long term survival rates remain poor, patients suffer from poor quality of life and more often than not patients are plagued by high rate of loco-regional as well as systemic recurrence. Survival rates in disease with regional spread have remained stagnant at 50 % over the past three decades in spite of developments in surgical and adjuvant therapy techniques.(45) Now more than ever, there is a need for newer strategies.

Increasingly more time and resources are being invested in the application of nutri-ceuticals for the management of malignancies. In the field of oral cancer, foremost among others is Curcumin. Alone or in combination with the standard chemotherapy agents, curcumin emerges as a promising therapeutic modality in the treatment of oral SCC.(36)

Numerous studies have been reported documenting the chemopreventive as well as chemotherapeutic effect of curcumin in oral SCC, some delving into the intrinsic molecular mechanisms involved with the same. In a study by Lotempio et al (46), the chemotherapeutic potential of curcumin was studied simultaneously in an *in vitro* model using three HNSCC cell lines (one of them being oral cavity cancer) along with *in vivo* xenograft model using athymic mice. In the xenograft model, curcumin was administered both as intratumoural injection as well as a topical paste. There was a modest reduction in the xenograft tumour growth, especially with topical curcumin application, from 0.08 cc in control arm to just under 0.06 cc in the experimental group, at the end of 22 days (25% reduction).

Cisplatin is the standard chemotherapeutic agent for advanced HNSCC, including oral cancer.(9) Although its main effect is systemic, to decrease the incidence of distant metastasis and hopefully improve long-term survival, it

does possess significant loco-regional action. The chemotherapeutic effect of curcumin was compared to cisplatin in a study by Ellatar and Viriji in 2000. (47) Oral cancer cells were developed *in vitro* and their growth suppression in response to curcumin, genistein and quercetin were compared with that of cisplatin. Both curcumin and cisplatin effectively suppressed tumour cell growth and proliferation in a dose-dependent fashion; however the potency of cisplatin in that study was five times that of curcumin.

The chemosensitising action of curcumin in combination with cisplatin for oral cancers has been analysed in literature. The study by Duarte, et al (35) has been mentioned earlier. With regard to the *in vivo* experiment, their results showed that in comparison to the control, there was a trend towards greater tumour inhibition with the curcumin-cisplatin combination (as evidenced by piece-wise regression model). However, the estimated difference in slopes of growth were not statistically significant ($P = 0.1098$). (35)

The study goes on to demonstrate that the tumour inhibitory function of cisplatin is mediated through activation of cellular senescence through p53 and p16 modulation. They were also able to show that the same p53 protein controlled the activation of NFκβ, a key molecular agent in cancer proliferation. As demonstrated by previous authors, the anticancer action of curcumin is probably through inhibition of NFκβ activity. The precise mechanism of this action was hypothesized as through direct inhibition of the protein Ikappa beta kinase (IKKβ). Thus, the chemopotentiation is believed to be due to simultaneous, mutually exclusive methods of inhibition of NFκβ transactivation.

Taking this further, Abuzeid et al (48) were able to develop a curcumin analogue, called FLLL32, which successfully sensitised cisplatin resistant oral SCC cell lines and increased the local action of cisplatin. Lower doses of cisplatin could be used to achieve similar oncological outcomes, as compared to monotherapy with cisplatin.

In the studies mentioned above, all experiments have been performed on xenograft tumours, produced by inoculation of cancer cell lines. In fact there is no study which assesses the chemopotentiating effect of curcumin on cisplatin in a tumour of native buccal mucosa, produced by chemical carcinognenesis.

The aim of our study was to first replicate an oral cancer model as comparable as possible to the natural human one, thereby create a multistep carcinogenetic process in the native oral mucosa. It was our hypothesis that such an *in vivo* model would better reflect the natural process as compared to a cell culture *in vitro* or xenograft model.

This was achieved with 7, 12 DMBA. Our induction protocol was based on a study by Heber, et al. (43). The authors compared five carcinogenesis protocols in the hamster cheek model using 7, 12 DMBA, involving varying periods of induction. These were twice-a-week application of the carcinogen for 4 weeks, 6 weeks, 7 weeks and 8 weeks respectively, and thrice-a-week for 12 weeks. Tumour formation was recorded at the end of the study period(T0) in more than

90% of animals for the 12 week protocol, one month after T0 in the 8 week protocol and at 3, 4 and 8 months after T0 in the 7 week, 6 week and 4 week protocols, respectively. This study establishes the 12 week protocol as an ideal method for short term follow up of carcinogenesis and experimentation of therapeutic modalities.

As earlier authors had demonstrated, in our study 100 % tumour incidence was achieved in the animals at the end of the 12 week induction period. This was deducted both by gross examination, as well as confirmed by histopathological examination of the control arm. In fact the entire spectrum, from papilloma, dysplasia, carcinoma *in situ* as well as invasive carcinoma was seen.

ANALYSIS OF TUMOUR REGRESSION WITH CURCUMIN ALONE.

In Group B, i.e. the animals who received curcumin as the sole

chemotherapeutic agent, there was demonstrable reduction in median tumour area from the pre- to post-intervention period. The absolute reduction in area was 54 mm^2, a 16.08 % reduction from the pre-intervention tumour area(A0). The median tumour volume in this group was 274.75 mm^3 as compared to 828.96 mm^3 in the control group.

As expected, the therapeutic effect of curcumin alone was far inferior to that of cisplatin alone, where the reduction in tumour area was 3 times that of curcumin.

LoTempio, et al used the topical route for delivery of curcumin, whereas in our study, we opted for the enteral route. Much work has been done on determining the optimal route of administration of curcumin. Due to its metabolic instability and poor solubility in aqueous solvents, its oral bioavailability is limited. Trials in humans have demonstrated high daily doses (>8g /day) required in order to attain therapeutic systemic levels of curcumin.(24) Higher doses may cause side-effects and hence preclude its use. This difficulty has been overcome by the conception of innovative methods of drug delivery. Incorporation of

curcumin into liposomal structures to enhance delivery has been tried, as in studies by Suh et al.(36) Modification of covalent bonding within the curcumin structure has led to the development of novel curcumin analogues with better solubility and subsequent better bioavailability. In a novel study by Lin, et al(49) curcumin was incorporated into lipid based microemulsions and administered to the test subjects. The release of the molecules from the emulsion was triggered by ultrasonogram signals. This provides the opportunity for targeted therapy, wherein the desired action of the drug is effected only at a specific site or target organ.

ANALYSIS OF TUMOUR REGRESSION WITH CURCUMIN ALONG WITH CISPLATIN.

Group D, i.e. animals that received combination therapy with curcumin and Cisplatin, demonstrated maximum tumour suppression as compared to Cisplatin alone. There was a 77.22% reduction in tumour area in this group, with a median tumour volume of 50 mm^3. This was significantly more suppressive

than in group C, where the area of tumour was reduced only by 49.82% and tumour volume was 85.30 mm^3. When analysed for statistical significance of differences between the groups, a significant difference was obtained (p 0.005). From our analysis we infer that the combination of therapies offers greater tumour suppression than either therapy alone. In fact the combination seems to be synergistic, with the total area reduction in Group D exceeding the sum of reduction of area of Group B and C (219 mm^2 vs 141mm^2)

FUTURE RESEARCH

It remains uncontested that curcumin is a valuable tool in the fight against oral cancer. As more light is being shed on its mysterious mechanisms of action, studies have proved its chemotherapeutic and chemopotentiating ability. It will be necessary to duplicate results in larger study populations of animals, as well as to progress to dose-response studies in animals. The next step would be to design experiments focussing on side effect profiling in order to arrive at a

suitable formula for combination of these drugs, while achieving the same tumour inhibition.

While curcumin may not be conceivable as the single chemotherapeutic agent of a regimen for oral squamous cell cancer, the hope is that equal, or possibly better, levels of tumour inhibition are obtained with lower doses of Cisplatin through combination therapy, as compared to monotherapy with Cisplatin.

LIMITATIONS

LIMITATIONS

The main limitation of this study remains the small number of animals permissible for study in each group, and thereby the possibility of inaccurate extrapolation of the results obtained. There were three cases of mortality among the study cases, which again could skew results considerably simply due to the small numbers at play.

CONCLUSION

CONCLUSION

1. Curcumin inhibits the growth of 7, 12 DMBA induced oral squamous cell cancer in hamsters.
2. Curcumin exhibits highly significant synergistic effect with Cisplatin in the inhibition of 7, 12 DMBA induced oral squamous cell cancer in hamsters.

BIBLIOGRAPHY

1. Scully C. Cancers of the Oral Mucosa. http://emedicine.medscape.com/article/1075729
2. Sturgis EM. A review of social and behavioral efforts at oral cancer preventions in India. *Head and Neck* ,2004,**26**(11), 937–944.
3. O'Sullivan EM. Oral and pharyngeal cancer in Ireland. Ir Med J. 2005 Apr;**98**(4):102-5.
4. International Classification of Diseases – 10, World Health Organisation. http://apps.who.int/classifications/icd10/browse/2010/en
5. Llewellyn CD, Johnson NW, Warnakulasuriya KAAS . *Oral Oncology* 2001; **37**, 401-418
6. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet].
   Lyon, France: International Agency for Research on Cancer; 2010.
   http://globocan.iarc.fr
7. Rodriguez T, Aliteri A, Chatenoud L, Gallus S, Bosetti C, Negri E, Franchesci S, Levi F, et al. Risk factors for oral and pharyngeal cancer in young adults. *Oral Oncology*,2004;**40** (2), 207-13
8. Scheifele C; Reichart PA; Hippler-Benscheidt M; Neuhaus P; Neuhaus R. Incidence of oral, pharyngeal, and laryngeal squamous cell carcinomas among 1515 patients after liver transplantation. *Oral Oncol*. 2005; **41**(7):670-6
9. Management of Head and Neck cancer. National Comprehensive Cancer Network Guidelines 2012.
10. Fan KH, Lin CY, Kang CJ, Huang SF, Wang HM, Chen EY, Chen IH, Liao CT, Cheng AJ, Chang JT. Combined-modality treatment for advanced oral tongue squamous cell carcinoma.
    Int J Radiat Oncol Biol Phys. 2007 Feb **1**;67(2):453-61.
11. Jones E. Side effects of Cisplatin. http://www.cisplatin.org
12. Neoplasia: Chapter 8, Robbins and Cotran Pathological Basis of Disease
13. http://www.ana-jana.org

14. Kalra EK. Nutraceutical - Definition and Introduction. AAPS PharmSci 2003; **5** (3) Article 25
15. Aggarwal BB, Sundaram C, Malani N, Ichikawa H. Curcumin: the Indian solid gold. *Adv Exp Med Biol*. 2007; **595:1**-75.
16. Aggarwal BB, Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. *Trends Pharmacol Sci*. 2009 Feb; **30**(2):85-94.
17. Aggarwal BB, Kuzhuvelil B. Harikumar. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *The International Journal of Biochemistry & Cell Biology*. January 2009, **41(**1), 40-59
18. Pan M, Huang TM, Lin J. Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug metabolism and disposition* , **27**, No. 1
19. Ireson CR, Donald J. Jones L,Orr S, et al. Metabolism of the Cancer Chemopreventive Agent Curcumin in Human and Rat Intestine. *Cancer Epidemiol Biomarkers Prev* 2002; 11:105-111.
20. P. Anand et al. Curcumin and cancer: An "old—age" disease with an "age-old" solution. *Cancer Letters* **267** (2008) 133–164.
21. Kim JH, Gupta SC, Park B, Yadav VR, Aggarwal BB. Turmeric (Curcuma longa) inhibits inflammatory nuclear factor (NF)-κB and NF-κB-regulated gene products and induces death receptors leading to suppressed proliferation, induced chemosensitization, and suppressed osteoclastogenesis. *Mol Nutr Food* Res. 2012 Mar;56(3):454-65.
22. Ravindran J,Prasad S, Aggarwal BB. Curcumin and Cancer Cells: How Many Ways Can Curry Kill Tumor Cells Selectively?. *AAPS J.* 2009 September; **11**(3): 495–510.
23. Balasubramanian S, Eckert RL. Keratinocyte proliferation, differentiation, and apoptosis-differential mechanisms of regulation by curcumin, EGCG and apigenin. Toxicol Appl Pharmacol. 2007;224:214–9
24. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res*. 2001 Jul-Aug;**21**(4B):2895-900.

25. R. Dantzer, K.W. Kelley, Twenty years of research on cytokine-induced sickness behavior, *Brain Behav. Immun*.**21** (2007) 153–160.
26. Aggarwal S, Takada Y, Singh S, Myers JN, Aggarwal BB. Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-jB signaling. *Int J Cancer*. 2004;**111**(5):679–92.
27. Sharma C, Kaur J, Shishodia S, Aggarwal BB, Ralhan R. Curcumin down regulates smokeless tobacco-induced NF-kappaB activation and COX-2 expression in human oral premalignant and cancer cells. *Toxicology*.2006;**228**(1):1–15.
28. Manoharan S, Balakrishnan S, Menon VP, Alias LM, Reena AR. Chemopreventive efficacy of curcumin and piperine during 7,12-dimethylbenz[a]anthraceneinduced hamster buccal pouch carcinogenesis. *Singapore Med J* 2009;**50**(2):139–46.
29. Chang KW, Hung PS, Lin IY, Hou CP, Chen LK, Tsai YM, et al. Curcumin upregulates insulin-like growth factor binding protein-5 (IGFBP-5) and C/ EBPalpha during oral cancer suppression. *Int J Cancer* 2010;**127**(1):9–20.
30. Lin YC, Chen HW, Kuo YC, Chang YF, Lee YJ, Hwang JJ. Therapeutic efficacy evaluation of curcumin on human oral squamous cell carcinoma xenograft using multimodalities of molecular imaging. *Am J Chin Med* 2010;**38**(2):343–58.
31. Li N, Chen X, Liao J, Yang G, Wang S, Josephson Y, et al. Inhibition of 7,12 dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin. *Carcinogenesis* 2002;**23**(8):1307–13.
32. Goel A and Aggarwal BB. Curcumin, the Golden Spice From Indian Saffron, Is Chemosensitizer and Radiosensitizer for Tumors and Chemoprotector and Radioprotector for Normal Organs. *Nutrition and Cancer* 2010, **62**:7, 919-930
33. Khafif A, Hurst R, Kyker K, Fliss DM, Gil Z, Medina JE. Curcumin: a new radio-sensitizer of squamous cell carcinoma cells. *Otolaryngol Head Neck Surg*. 2005 Feb;**132**(2):317-21.
34. López-Jornet P, Camacho-Alonso F, Gómez-Garcia F. Effect of curcumin and irradiation in PE/CA-PJ15 oral squamous cell carcinoma. Acta Odontol Scand. 2011 Sep;69(5):269-73.
35. Suh JD, Duarte V et al. R424 – Suppression of HNSCC with Liposomal Curcumin and Cisplatin . *Otolaryngology - Head and Neck Surgery* **139**,(2) :1:185-186
36. Duarte VM, Han E, Veena MS, Salvado A, Suh JD, Liang LJ, et al. Curcumin enhances the effect of cisplatin in suppression of head and neck squamous cell

carcinoma via inhibition of IKKb protein of the NFkB pathway. *Mol Cancer Ther*. 2010;**9**(10):2665–75.

37. Kuhad A, Pilkhwal S, Sharma S, Tirkey N, and Chopra K: Effect of curcumin on inflammation and oxidative stress in cisplatin-induced experimental nephrotoxicity. *J Agric Food Chem* 55, 10150–10155, 2007.
38. Golden hamsters : en.wikipedia.org/wiki/Golden_hamster
39. Neoplasia: Chapter 8, Robbins and Cotran Pathological Basis of Disease
40. Animal models of cancer : emice.nci.nih.gov/aam
41. 7,12 Dimethylbenzanthracene : en.wikipedia.org/wiki/7,12 Dimethylbenzanthracene
42. 7,12 Dimethylbenzanthracene : Product catalogue and safety sheet, Sigma—Aldrich India. www.sigmaaldrich.com/catalog/product/sigma/d3254?lan=en&region=IN
43. Heber EM, Monti Hughes A, Pozzi EC, Itioz ME et al. Development of a model of tissue with potential malignant disorders(PMD) in the hamster cheek pouch to explore the long-term potential therapeutic and/or toxic effects of different therapeutic modalities. *Arch Oral Biol.*2010 Jan;**55**(1):46--51
44. Standard Operating Protocol for 7,12 Dimethylbenzanthracene, UC Irvine Environmental Health & Safety.
45. Surveillance Epidemiology and End Results(SEER) database, United States National Cancer Institute. seer.cancer.gov/statfacts/html/oralcav/html
46. LoTempio MM, Veena MS, Steele HL, Ramamurthy B, Ramalingam TS, Cohen AN, et al. Curcumin suppresses growth of head and neck squamous cell carcinoma. *Clin Cancer Res* 2005**;11**(19 Pt 1):6994–7002.
47. Elattar TM, Virji AS. The inhibitory effect of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells in-vitro. *Anticancer Res*. 2000;**20**(3A):1733–8.
48. Abuzeid WM, Davis S, Tang AL, Saunders L, Brenner JC, Lin J, et al. Sensitization of head and neck cancer to cisplatin through the use of a novel curcumin analog. *Arch Otolaryngol Head Neck Surg* 2011;**137**(5):499–507.
49. Lin HY, Thomas JL, Chen HW, Shen CM, Yang WJ, Lee MH. In vitro suppression of oral squamous cell carcinoma growth by ultrasound-mediated delivery of curcumin microemulsions. *Int J Nanomedicine* 2012;7:941–51.

ANNEXURES

1. TURN-IT-IN ANTIPLAGIRISM SOFTWARE RECEIPT AND ORIGINALITY CERTIFICATE
2. EXCEL WORKSHEET OF DATA ENTRY